Chapter 1

Lignocellulose Biodegradation and Applications in Biotechnology

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Lignocellulosic biomass such as agricultural and forestry residues and herbaceous energy crops can serve as low cost feedstocks for production of fuel ethanol and other value-added commodity chemicals. However, development of efficient pretreatment and cost-effective enzymatic conversion of any lignocellulosic biomass to fermentable sugars is a key issue. In this overview chapter, various pretreatment options (dilute acid, steam explosion, alkaline peroxide) and enzymes (mainly cellulases and hemicellulases) involved in lignocellulose degradation are presented. Mixed sugars generated by lignocellulose biodegradation are fermented to fuel ethanol, xylitol, 2-3-butanediol and other value-added products. Recent advances in the developments on lignocellulose biodegradation and applications in biotechnology are reviewed.

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In 2003, about 2.81 billion gallons of ethanol are produced annually in the United States, with approximately 95% derived from fermentation of corn starch. With increased attention to clean air and oxygenates for fuels, opportunities exist for rapid expansion of the fuel ethanol industry. Various lignocellulosic biomass such as agricultural residues, wood, municipal solid wastes and wastes from pulp and paper industry can serve as low cost and abundant feedstocks for production of fuel ethanol or value-added chemicals. It is estimated that approximately 50 billion gallons of ethanol could be produced from current biomass wastes with the potential to produce up to 350 billion gallons from dedicated energy farms in the USA (*1*). At present, the degradation of lignocellulosic biomass to fermentable sugars represents significant technical and economic challenges, and its success depends largely on the development of highly efficient and cost-effective enzymes for conversion of pretreated lignocellulosic substrates to fermentable sugars. In this overview chapter, the author reviews the current knowledge on lignocellulose biodegradation and use of lignocellulosic hydrolyzates as feedstocks for developing bio-based products and processes.

Structure and Composition of Lignocellulosic Biomass

Lignocellulosic biomass includes various agricultural residues (straws, hulls, stems, stalks), deciduous and coniferous woods, municipal solid wastes (MSW, paper, cardboard, yard trash, wood products), waste from pulp and paper industry and herbaceous energy crops (switchgrass, barmudagrass). The compositions of these materials vary. The major component is cellulose (35-50%), followed by hemicellulose (20-35%) and lignin (10-25%). Proteins, oils and ash make up the remaining fraction of lignocellulosic biomass (*1*). The structures of these materials are complex with recalcitrant and heterogeneous characteristics and native lignocellulose is resistant to an enzymatic hydrolysis. In the current model of the structure of lignocellulose, cellulose fibers are embedded in a lignin-polysaccharide matrix. Xylan may play a significant role in the structural integrity of cell walls by both covalent and non-covalent associations (*2*).

Cellulose is a linear polymer of D-glucose units linked by 1,4-ß-D-glucosidic bonds. Hemicelluloses are heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids. Unlike cellulose, hemicelluloses are not chemically homogeneous. Hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses contain mostly glucomannans (*3*). Xylans of many plant materials are heteropolysaccharides with homopolymeric backbone chains of 1,4-linked β-D-xylopyranose units. Besides xylose, xylans may contain arabinose, glucuronic acid or its 4-*O*-methyl ether, and acetic, ferulic and *p*-coumaric acids. The frequency and composition of branches are dependent on the source of xylan (*4*). The backbone consists of *O*-acetyl, α-L-arabinofuranosyl, α-1,2-linked glucuronic or 4-*O*-methylglucuronic acid substituents. However, unsubstituted linear xylans have also been isolated from guar seed husk, esparto grass and tobacco stalks (*5*).

Xylans can thus be categorized as linear homoxylan, arabinoxylan, glucuronoxylan and glucuronoarabinoxylan.

Xylans from different sources, such as grasses, cereals, softwood and hardwood, differ in composition. Birch wood (Roth) xylan contains 89.3 % xylose, 1% arabinose, 1.4% glucose and 8.3% anhydrouronic acid (*6*). Rice bran neutral xylan contains 46% xylose, 44.9% arabinose, 6.1% galactose, 1.9% glucose and 1.1% anhydrouronic acid (*7*). Wheat arabinoxylan contains 65.8% xylose, 33.5% arabinose, 0.1% mannose, 0.1% galactose and 0.3% glucose (*8*). Corn fiber xylan is one of the complex heteroxylans containing β-(1,4)-linked xylose residues (*9*). It contains 48-54% xylose, 33-35% arabinose, 5-11% galactose and 3-6% glucuronic acid (*10*). About 80% of the xylan backbone is highly substituted with monomeric side-chains of arabinose or glucuronic acid linked to *O*-2 and/or *O*-3 of xylose residues and also by oligomeric side chains containing arabinose, xylose and sometimes galactose residues (*11*). The heteroxylans, which are highly cross-linked by diferulic bridges, constitute a network in which the cellulose microfibrils may be imbedded (*12*). Structural wall proteins might be cross-linked together by isodityrosine bridges and with feruloylated heteroxylans, thus forming an insoluble network (*13*). Ferulic acid is covalently cross-linked to polysaccharides by ester bonds and to components of lignin mainly by ether bonds (*14*). In softwood heteroxylans, arabinofuranosyl residues are esterified with *p*-coumaric acids and ferulic acids (*15*). In hardwood xylans, 60-70% of the xylose residues are acetylated (*16*). The degree of polymerization of hardwood xylans (150-200) is higher than that of softwoods (70-130).

Pretreatment of Lignocellulosic Biomass

The pretreatment of any lignocellulosic biomass is crucial before enzymatic hydrolysis. The objective of pretreatment is to decrease the crystallinity of cellulose which enhances the hydrolysis of cellulose by cellulases (*17*). Various pretreatment options are available to fractionate, solubilize, hydrolyze and separate cellulose, hemicellulose and lignin components (*1, 18-20*). These include concentrated acid (*21*), dilute acid (*22*), SO_2 (*23*), alkali (*24, 25*), hydrogen peroxide (*26*), wet-oxidation (*27*), steam explosion (autohydrolysis) (*28*), ammonia fiber explosion (AFEX) (29), CO_2 explosion (*30*), liquid hot water (*31*) and organic solvent treatments (*32*). In each option, the biomass is reduced in size and its physical structure is opened. Some methods of pretreatment of Lignocellulose is given in Table I.

The effectiveness of dilute acids to catalyze the hydrolysis of hemicellulose to its sugar components is well known . Two categories of dilute acid pretreatment are used: High temperature (> 160°C) continuous-flow for low solids loading (5-10%, w/w) and low temperature (< 160°C) batch process for high solids loading (10-40%, w/w) (*33*). Dilute acid pretreatment at high temperature usually hydrolyzes hemicellulose to its sugars (xylose, arabinose and other sugars) that are water

Table I. Methods for pretreatment of lignocellulosic biomass

Method	*Example*
Autohydrolysis	Liquid hot water, steam pressure, steam explosion, supercritical CO_2explosion
Acid treatment	Dilute acid (H_2SO_4), Concentrated acid (H_2SO_4)
Alkali treatment	Sodium hydroxide, lime, ammonia, alkaline hydrogen peroxide
Organic solvent with water	Methanol, ethanol, butanol, phenol

soluble (*18*). The residue contains cellulose and often much of the lignin. The lignin can be extracted with solvents such as ethanol, butanol, or formic acid. Alternatively, hydrolysis of cellulose with lignin present produces water-soluble sugars and the insoluble residues that are lignin plus unreacted materials. Torget et al. (*34*) achieved both high xylan recovery and high simultaneous saccahrification and fermentation (SSF) conversion while applying extremely dilute H_2SO_4 (0.07 wt %) in a counter-current flowthrough configuration. A major problem associated with the dilute acid hydrolysis of lignocellulosic biomass is the poor fermentability of the hydrolyzates. A drawback of the concentrated acid process is the costly recovery of the acid.

Steam explosion provides effective fractionation of lignocellulosic components at relatively low costs (*35*). Optimal solubilization and degradation of hemicellulose are generally achieved by either high temperature and short residence time (270°C, 1 min) or lower temperature and longer residence time (190°C, 10 min) steam explosion (*36*). The use of SO_2 as a catalyst during steam pretreatment results in the enzymatic accessibility of cellulose and enhanced recovery of the hemicellulose derived sugars (*37*). Steam pretreatment at 200-210°C with the addition of 1% SO_2 (w/w) was superior to other forms of pretreatment of willow (*38*). A glucose yield of 95%, based on the glycan available in the raw material, was achieved. Steam explosion can induce hemicellulose degradation to furfural and its derivatives and modification of the lignin-related chemicals under high severity treatment (> 200°C, 3-5 min, 2-3% SO_2) (*39*). Boussaid et al. (*40*) recovered around 87% of the original hemicellulose component in the water-soluble stream by steam explosion of Douglas fir softwood under low severity conditions (175°C, 7.5 min, 4.5% SO_2). More than 80% of the recovered hemicellulose was in monomeric form. Enzymatic digestibility of the steam-exploded Douglas-fir wood chips (105°C, 4.5 min, 4.5% SO_2) was significantly improved using an optimized alkaline peroxide treatment (1% H_2O_2, pH 11.5 and 80°C, 45 min) (*41*). About 90% of the lignin in the original wood was solubilized by this procedure, leaving a cellulose-rich residue that was completely hydrolyzed within 48 h, using an enzyme (cellulase) loading of 10FPU/g cellulose. Saccharification of 100 g sugarcane bagasse with enzymes after steam explosion with 1% H_2SO_4 at 220°C for 30 sec at water to solid ratio of 2:1 yielded 65.1g sugar (*42*).

A pretreatment method involves steeping of the lignocellulosic biomass (using corn cob as a model feedstock) in dilute NH_4OH at ambient temperature to remove lignin, acetate and extractives (*43*). This is followed by dilute acid treatment that readily hydrolyzes the hemicellulose fraction to simple sugars, primarily xylose. The residual cellulose fraction of biomass can then be enzymatically hydrolyzed to glucose. Sugarcane bagasse, corn husk and switchgrass were pretreated with ammonia water to enhance enzymatic hydrolysis (*44*). Garrote et al. (*45*) treated *Eucalyptus* wood substrates with water under selected operational conditions (autohydrolysis reaction) to obtain a liquid phase containing hemicellulose decomposition products (mainly acetylated xylooligosaccharides, xylose and acetic acid). In a further acid catalyzed step (posthydrolysis reaction), xylooligosaccharides were converted into xylose. Wet oxidation method can be used for fractionation of lignocellulosics into solubilized hemicellulose fraction and a solid cellulose fraction susceptible to enzymatic saccharification. Bjerre et al. (*46*) found that combination of alkali and wet oxidation did not generate furfural and 5-hydroxymethyl furfural (HMF). Klinke et al. (*47*) characterized the degradation products from alkaline wet oxidation (water, sodium carbonate, oxygen, high temperature and pressure) of wheat straw. Apart from CO_2 and water, carboxylic acids were the main degradation products from hemicellulose and lignin. Aromatic aldehyde formation was minimized by the addition of alkali and temperature control. Oxygen delignification of kraft pulp removed up to 67% of the lignin from softwood pulp and improved the rate and yield from enzymatic hydrolysis by up to 111% and 174%, respectively (*48*). Palm and Zacchi (*49*) extracted 12.5 g of hemicellulose oligosaccharides from 100 g of dry spruce using a microwave oven at 200°C for 5 min.

Supercritical CO_2 explosion was found to be effective for pretreatment of cellulosic materials before enzymatic hydrolysis (*50, 51*). Zheng et al. (*52*) compared CO_2 explosion with steam and ammonia explosion for pretreatment of sugarcane bagasse and found that CO_2 explosion was more cost-effective than ammonia explosion and did not cause the formation of inhibitory compounds that could occur in steam explosion.

Phenolic compounds from lignin degradation, furan derivatives (furfural and HMF) from sugar degradation and aliphalic acids (acetic acid, formic acid and levulinic acid) are considered to be fermentation inhibitors generated from pretreated lignocellulosic biomass (*53*). The formation of these inhibitors depends on the process conditions and the lignocellulosic feedstocks (*54*). Various methods for detoxification of the hydrolyzates have been developed (*55*). These include treatment with ion-exchange resins, charcoal or ligninolytic enzyme laccase, pre-fermentation with the filamentous fungus *Trichoderma reesei*, removal of non-volatile compounds, extraction with ether or ethyl acetate and treatment with alkali (lime) or sulfite. Treatment with alkali (overliming) has been widely used for detoxification of lignocellulosic hydrolyzates prior to alcohol fermentation. However, overliming is a costly method which also produces low-value byproducts such as gypsum (*56*). Softwood hydrolyzate, when overlimed with wood ash, improved its fermentability to ethanol which is due to the reduction of the inhibitors such as furan and phenolic compounds and to nutrient effects of some inorganic components from the wood ash on the fermentation (57). Persson et al. (*58*) employed countercurrent flow supercritical fluid extraction to detoxify a dilute acid hydrolyzate of spruce prior to ethanol fermentation with baker's yeast. Weil et al. (*59*) developed a method for the removal of furfural from biomass hydrolyzate by using a polymeric adsorbent, XAD-4, and desorption of the furfural to regenerate the adsorbent using ethanol. Bjorklund et al. (*60*) explored the possibility of using lignin residue left after acid hydrolysis of lignocellulosic material for detoxification of spruce dilute acid hydrolyzates prior to fermentation with *Saccharomyces cerevisiae*. Treatment with the lignin residue removed up to 53% of the phenolic compounds and up to 68% of the furan aldehydes in a spruce dilute acid hydrolyzate. Up to 84% of the lignin-derived compounds can be extracted with organic solvents (ethyl acetate and diethyl ether) from *Eucalyptus* wood acid hydrolyzate (*61*). The phenolic compounds extracted by solvents showed antioxidant activity.

Each pretreatment method offers distinct advantages and disadvantages. The pretreatment of lignocellulosic biomass is an expensive procedure with respect to cost and energy.

Cellulose Biodegradation

Effective hydrolysis of cellulose to glucose requires the cooperative action of three enzymes: endo-1, 4-ß-glucanase (EC 3.2.1.4), exo-1, 4-ß-glucanase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21). Cellulolytic enzymes with ß-glucosidase act sequentially and cooperatively to degrade crystalline cellulose to glucose. Endoglucanase acts in a random fashion on the regions of low crystallinity of the cellulosic fiber whereas exoglucanase removes cellobiose (ß-1, 4 glucose dimer) units from the non-reducing ends of cellulose chains. Synergism between these two enzymes is attributed to the *endo-exo* form of cooperativity and has been studied extensively between cellulases in *T. reesei* in the degradation of cellulose (*62*). Besides synergism, the adsorption of the cellulases on the insoluble substrates is a necessary step prior to hydrolysis. Cellobiohydrolase appears to be the key enzyme for the degradation of native cellulose (*63*). The catalytic site of the enzyme is covered by long loops, resulting in tunnel morphology (*64*). The loops can undergo large movements, leading to the opening or closing of the tunnel roof (*65*). An endo type attack of the polymeric substrates becomes possible when the roof is open and once entrapped inside the catalytic tunnel, a cellulose chain is threaded through the tunnel and sequentially hydrolyzed one cellobiosyl unit at a time. Kleywegt et al. (*66*) revealed the presence of shorter loops that create a groove rather than a tunnel in the structure of the enzyme EGI from *T. reesei.* In most organisms, cellulases are modular enzymes that consist of a catalytic core connected to a cellulose-binding domain (CBD) through a flexible and heavily glycosylated linker region (*67*). The CBD is responsible for bringing the catalytic domain in an appropriate position for the breakdown of cellulose. Binding of cellulases and the formation of cellulose-cellulase complexes are considered critical steps in the hydrolysis of insoluble cellulose (*68*). ß-Glucosidase hydrolyzes cellobiose and in some cases cellooligosaccharides to glucose. The enzyme is generally responsible for the regulation of the whole cellulolytic process and is a rate limiting factor during enzymatic hydrolysis of cellulose as both endoglucanase and cellobiohydrolase activities are often inhibited by cellobiose (*69-71*). Thus, β-glucosidase not only produces glucose from cellobiose but also reduces cellobiose inhibition, allowing the cellulolytic enzymes to function more efficiently. However, like ß-glucanases, most ß-glucosidases are subject to end-product (glucose) inhibition (*72*). *C. peltata* produces a highly glucose tolerant β-glucosidase with a K_i value of 1.4 M (252 mg/ml) for glucose (*73*). The kinetics of the enzymatic hydrolysis of cellulose including adsorption, inactivation and inhibition of enzymes have been studied extensively (*74*). For a complete hydrolysis of cellulose to glucose, the enzyme system must contain the three enzymes in right proportions. *T. reesei* (initially called *T. viride*) produces at least five endoglucanases (EGI, EGII, EGIII, EGIV and EGV), two exoglucanases (CBHI and CBHII) and two β-glucosidases (BGLI and BGLII) (*75*). An exo-exo synergism between the two cellobiohydrolases was also observed (*76*). The fungus produces up to 0.33 g protein per g of utilizable carbohydrate (*77*).

Product inhibition, thermal inactivation, substrate inhibition, low product yield and high cost of cellulase are some barriers to commercial development of the enzymatic hydrolysis of cellulose. Many microorganisms are cellulolytic. However, only two microorganisms (*Trichoderma* and *Aspergillus*) have been studied extensively for cellulase. A newly isolated *Mucor circinelloides* strain produces a complete cellulase enzyme system (*78*). The endoglucanase from this strain was found to have a wide pH stability and activity. There is an increasing demand for the development of thermostable, environmentally compatible, product and substrate tolerant cellulases with increased specificity and activity for application in the conversion of cellulose to glucose in the fuel ethanol industry. Thermostable cellulases offer certain advantages such as higher reaction rate, increased product formation, less microbial contamination, longer shelf-life, easier purification and better yield.

The cellulose hydrolysis step is a significant component of the total production cost of ethanol from wood (*79*). Achieving a high glucose yield is necessary (>85% theoretical) at high substrate loading (>10% w/v) over short residence times (<4 days). Simultaneous saccharification (hydrolysis) of cellulose to glucose and fermentation of glucose to ethanol (SSF) improve the kinetics and economics of biomass conversion by reducing accumulation of hydrolysis products that are inhibitory to cellulase and β-glucosidase, reducing the contamination risk because of the presence of ethanol, and reducing the capital equipment requirements (*80*). An important drawback of SSF is that the reaction has to operate at a compromised temperature of around 30°C instead of enzyme optimum temperature of 45-50°C. Enzyme recycling, by ultrafiltration of the hydrolyzate, can reduce the net enzyme requirement and thus lower costs (*81*). A preliminary estimate of the cost of ethanol production for SSF technology based on wood-to-ethanol process is $1.22/gal of which the wood cost is $0.459/gal (*82*). A separate fungal enzyme hydrolysis and fermentation process for converting lignocellulose to ethanol were also evaluated (*83*). The cellulase enzyme was produced by the fungal mutant *Trichoderma* Rut C-30 (the first mutant with greatly increased β-glucosidase activity) in a fed batch production system that is the single most expensive operation in the process. The conversion of lignocellulosic biomass to fermentable sugars requires the addition of complex enzyme mixtures tailored for the process and parallel reuse and recycle the enzymes until the cost of enzymes comes down. Enzyme recycling may increase the rates and yields of hydrolysis, reduce the net enzyme requirements and thus lower costs (*84*). As mentioned earlier, the first step in cellulose hydrolysis is considered as the adsorption of cellulase onto cellulosic substrate. As the cellulose hydrolysis proceeds, the adsorbed enzymes (endo- and exo-glucanase components) are gradually released in the reaction mixture. The β-glucosidase does not adsorb onto the substrate. These enzymes can be recovered and reused by contacting the hydrolyzate with the fresh substrate. However, the amount of enzyme recovered is limited because some enzymes remain attached to the residual substrate, and some enzymes are thermally inactivated during hydrolysis. Poor recovery of cellulase was achieved in the case of substrates containing a high proportion of lignin (*85*). Addition of surfactant to enzymatic hydrolysis of lignocellulose increases the conversion of cellulose to soluble sugars. Castanon and Wilke (*86*) reported a 14% increase in glucose yield and more than twice as much recovered enzyme from newspaper saccharification when Tween 80 was added. Karr and Holtzapple (*87*) studied the effect of Tween on the enzymatic hydrolysis of lime pretreated corn stover and concluded that Tween improves corn stover hydrolysis through three effects: enzyme stabilizer, lignocellulose disrupter and enzyme effector. The enhancement is due to reduction of the unproductive enzyme adsorption to the lignin part of the substrate as a result of hydrophobic interaction of surfactant with lignin on the lignocellulose surface, which releases nonspecifically bound enzyme (*88*).

Cellolignin is an industrial residue obtained during the production of furfural from wood and corn cobs when pretreated by dilute H_2SO_4 at elevated temperature. It was completely converted to glucose by cellulase from *T. viride* and *A. foetidus* (*89*). The concentration of glucose in the hydrolyzate reached 4-5.5% with about 80% cellulose conversion. Kinetic analysis of cellolignin hydrolysis, using a mathematical model of the process, has shown that, with product inhibition, nonspecific adsorption of cellulase onto lignin and substrate induced inactivation seem to affect negatively the hydrolysis efficiency. Borchert and Buchholz (*90*) investigated the enzymatic hydrolysis of different cellulosic materials (straw, potato pulp, sugar beet pulp) with respect to reactor design. The kinetics were studied including enzyme adsorption, inhibition and inactivation. The results suggest the use of reactors with plug flow characteristics to achieve high substrate and product concentrations and to avoid back-mixing to limit the effect of product inhibition. For efficient use of cellulases, a reactor with semipermeable hollow fiber or an ultrafilter membrane was used, and this allowed cellulases to escape end-product inhibition (*91-94*). A totally integrated biotechnology of rice straw conversion into ethanol was reported (*95*). It dealt with (a) ethanol refining of rice straw to segregate cellulose from pentose sugars and lignin, (b) preparation of highly active mixed cellulase enzymes, (c) a novel reactor system allowing rapid product formation involving enzymatic hydrolysis of cellulose to sugars followed by microbial conversion of the later into ethanol and its simultaneous flash separation employing a programmed recompression of ethanol vapors and condensation and (d) concentration of ethanol via alternative approaches. Use of cellulase enzymes improves ink detachment from old newspapers giving similar or better results in place of classical chemicals (*96*).

Hemicellulose Biodegradation

Hemicellulases are either glycosyl hydrolases or carbohydrate esterases. The total biodegradation of xylan requires endo-β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37) and several accessory enzymes, such as α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.131), acetylxylan esterase (EC 3.1.1.72), ferulic acid esterase (EC 3.1.1.73) and *p*-coumaric acid esterase, which are necessary for hydrolyzing various substituted xylans (*97*). The endo-xylanase attacks the main chains of xylans and β-xylosidase hydrolyzes xylooligosaccharides to xylose. The α-arabinofuranosidase and α-glucuronidase remove the arabinose and 4-*O*-methyl glucuronic acid substituents, respectively, from the xylan backbone. The esterases hydrolyze the ester linkages between xylose units of the xylan and acetic acid (acetylxylan esterase) or between arabinose side chain residues and phenolic acids, such as ferulic acid (ferulic acid esterase) and *p*-coumaric acid (*p*-coumaric acid esterase). It is stated that hindrance of lignocellulose biodegradation is associated with phenolic compounds (*98*).The phenolic acids are produced via the phenylpropanoid biosynthetic pathway (*99*). They act as a cross-linking agent between lignin and carbohydrates or between carbohydrates. β-Mannanase (EC 3.2.1.78) hydrolyzes mannan-based hemicellulases and liberate β-1,4-manno-oligomers, which can be further degraded to mannose by β-mannosidase (EC 3.2.1.25).

Many microorganisms, such as *Penicillium capsulatum* and *Talaromyces emersonii*, possess complete xylan degrading enzyme systems (*100*). Significant synergistic interactions were observed among endo-xylanase, β-xylosidase, α-arabinofuranosidase and acetylxylan esterase of the thermophilic actinomycete *Thermomonospora fusca* (*101*). Synergistic action between depolymerizing and side-group cleaving enzymes has been verified using acetylated xylan as a substrate (*102*). Many xylanases do not cleave glycosidic bonds between xylose units which are substituted. The side chains must be cleaved before the xylan backbone can be completely hydrolyzed (*103*). On the other hand, several accessory enzymes only remove side chains from xylooligosaccharides. These enzymes require a partial hydrolysis of xylan before the side chains can be cleaved (*104*). Although the structure of xylan is more complex than cellulose and requires several different enzymes with different specificities for complete hydrolysis, the polysaccharide does not form tightly packed crystalline structures like cellulose and is, thus, more accessible to enzymatic hydrolysis (*105*).

Corn fiber, a byproduct of corn wet milling facility, contains about 20% starch in addition to 15% cellulose and 35% hemicellulose (*106*). The xylan from corn fiber is highly resistant to enzymatic degradation by commercially available hemicellulases (*22*). Dilute acid (1% H_2SO_4 v/v, 15% solids) pretreatment at a relatively low temperature (120°C, 1 h) to minimize the formation of inhibitory compounds, followed by enzymatic saccharification of the cellulosic portion, is an excellent workable process for generating fermentable sugars (85-100% yield) from corn fiber (*22*). A partial saccharification of corn fiber was achieved using a crude enzyme preparation from *Aureobasidium* sp. (*107*). Christov et al. (*108*) showed that crude enzyme preparation from *A. pullulans* was only partially effective in the removal of xylan from dissolving pulp. Two newly isolated fungal cultures (*Fusarium proliferatum* NRRL 26517, *F. verticillioides* NRRL Y-26518) have the capability to utilize corn fiber xylan as growth substrate (*109-112*). The crude enzyme preparations from these fungi were able to degrade corn fiber xylan well but the purified endo-xylanases could not degrade corn fiber xylan. The purified β-xylosidase released xylose from xylobiose and other short-chain xylooligosaccharides. For effective hydrolysis of xylan substrates, a proper mix of endo-xylanase with several accessory enzymes is essential. *A. pullulans* produces a highly thermostable novel extracellular α-L-arabinofuranosidase that has the ability to rapidly hydrolyze arabinan and debranched arabinan and release arabinose from various arabinoxylans (*113*). Arabinose-rich lignocellulosic hydrolyzates can be used for production of the enzyme (*114*). Spagnuolo et al. (*115*) reported that incubation of beet pulp with α-L-arabinofuranosidase and end-arabinase produced a hydrolyzate consisting mainly arabinose.

Ferulic acid esterase breaks the ester linkage between ferulic acid and the attached sugar and release ferulic acid from complex cell walls such as wheat bran, sugar beet pulp, barley spent grain and oat hull (*116-119*). The ability of *Thermomyces lanuginosus* to produce high levels of cellulase-free thermostable xylanase has made the fungus an attractive source of the enzyme with potential as a bleach-boosting agent in the pulp and paper industry and as an additive in the baking industry (*120*).

Lignin Biodegradation

Lignin is a long chain heterogeneous aromatic polymer (average molecular weight 8,000-11,000) composed largely of phenylpropane units most commonly linked by ether bonds. It effectively protects the woody plants against microbial attack and only a few organisms including rot-fungi and some bacteria can degrade it (*121*). The conversion of cellulose and hemicellulose to fuels and chemicals will generate lignin as a by-product that can be burned to provide heat and electricity, converted to low-molecular weight chemicals and used in the manufacture of various polymeric materials. As lignin makes up 15-25% in some lignocellulosic biomass, the selling price of lignin has a very large impact on ethanol price (*35*).

Efficient removal of lignin from lignin-carbohydrate complex (LCC) is important in pulp and paper industry. The lignin barrier can be disrupted by a variety of pretreatment rendering the cellulose and hemicellulose more susceptible to enzymatic attack (*122*). There are many papers about microbial breakdowns of lignin, the enzymes and the pathways (*123-126*). Several white rot fungi have the ability to delignify kraft pulp. Their lignin-degrading capacity is attributed to extracellular oxidative enzymes that function together with low molecular weight cofactors (*127*). The degradation of lignin by the white rot fungus (WRF) *Phanerochaete chrysosporium* is catalyzed by extracellular peroxidases (lignin peroxidase, LiP, EC 1.11.1.14 and manganese peroxidase, MnP, EC 1.11.1.13) in a H_2O_2-dependent process (*128, 129*). LiP seems to use veratryl cation radical as mediator (*130*). The WRF *Ceriporiopsis subvermispora* produces laccase (EC 1.10.3.2) and MnP isozymes, as well as hemicellulases and a poor complex of cellulases lacking in cellobiohydrolase activity (*131*). Laccase is a family of 'blue-copper' oxidases containing four copper ions. It oxidizes the phenolic but not the non-phenolic subunits of lignin. Redox mediators drive laccase towards the oxidation of non-phenolic subunits, particularly the benzyl alcohol groups. Each laccase may have a preferred low molecular mass mediator substrate, which may represent a major secreted metabolite (*130*). A laccase and mediators with NO, NOH or HRNOH groups can be combined in a laccase-mediated system (Lignozyme process) that are effective in delignifying wood in a pilot pulp and paper process. A pre-oxidation of the α-hydroxy-β-arylether subunits in wood pulp by the laccase/violuric acid system appears to be promising for weakening the network of lignin, thereby activating it towards subsequent oxydelignification treatments (*132*). It was demonstrated that the WRF *Pycnoporus cinnabarinus* degrades lignin in the absence of both Lip and MnP (*133*). The fungus was found to produce predominantly laccase, and neither LiP nor MnP was produced. The factors involved in lignin biodegradation process are not yet fully understood (*134*).

The biodegradation of lignocellulose by *Rigidoporus lignosus* was greatly stimulated in solid state cultivation when compared with liquid culture (*135*). This WRF produces laccase and MnP. The edible mushroom *Pleurotus ostreatus* degrades lignin efficiently and selectively (*136*). It can, therefore, serve to upgrade lignocellulosic wastes. Lignin degradation by *Agaricus bisporus* accounts for a 30% increase in bioavailable holocellulose during cultivation on compost (*137*). Table II lists some of the enzymes involved in lignocellulose degradation.

Direct Microbial Conversion

In direct microbial conversion of lignocellulosic biomass into ethanol that could simplify the ethanol production process from these materials and reduce ethanol production costs, *Clostridium thermocellum*, a thermoanaerobe was used for enzyme production, hydrolysis and glucose fermentation (*138*). Cofermentation with *C. thermosaccharolyticum* simultaneously converted the hemicellulosic sugars to ethanol. However, the formations of by-products such as acetic acid and low ethanol tolerance are some drawbacks of the process. *Neurospora crassa* produces extracellular cellulase and xylanase and has the ability to ferment cellulose to ethanol (*139*).

In nature, cellulosic materials are degraded with the cooperation of many microorganisms. A mixed culture of one cellulolytic bacterium together with another non-cellulolytic bacterium was found to be effective for cellulose degradation (*140, 141*). Recently, Haruta et al. (*142*) obtained a microbial community from rice straw compost that had the capability of degrading 60% of rice straw within 4 days at 50°C. The community structure consisting of both aerobic and anaerobic bacteria remained constant after multiple subcultures exceeding 2 years.

Table II. Enzymes involved in lignocellulose degradation

Enzyme	Systematic name	EC number	Mode of action
Endo-1,4-β-glucanase	1,4-β-D-Glucan-4-glucanohydrolase	3.2.1.4	Endo-hydrolysis of 1,4-β-D-glucosidic linkages
Exo-1,4-β-glucanase	1,4-β-D-Glucan cellobiohydrolase	3.2.1.91	Hydrolysis of 1,4-β-D-glucosidic linkages releasing cellobiose
β-Glucosidase	β-D-Glucoside glucohydrolase	3.2.1.21	Hydrolyzes cellobiose and short chain cello-oligosaccharides to glucose
Endo-1,4-β- xylanase	1,4-β-D-Xylan xylanohydrolase	3.2.1.8	Hydrolyzes mainly interior β-1,4-xylose linkages of the xylan backbone
α-L-Arabinofuranosidase	α-L-Arabinofuranoside arabinofurano-hydrolase	3.2.1.55	Hydrolyzes terminal nonreducing α-arabinofuranose fromarabinoxylans
α-Glucuronidase	α-Glucuronoside glucanohydrolase	3.2.1.31	Releases glucuronic acid from glucuronoxylans
Acetylxylan esterase	Acetyl-ester acetylhydrolase	3.1.1.6	Hydrolyzes acetylester bonds in acetyl xylans
Ferulic acid esterase	Carboxylic ester hydrolase	3.1.1.1	Hydrolyzes feruloylester bonds in xylans
Lignin peroxidase		1.11.1.7	Oxidation of benzilic alcohols, cleavage of C-C bonds, cleavage of C-O bonds.
Manganese peroxidase		1.11.1.7	Catalytically dependent on H_2O_2 and Mn^{2+} ions
Laccase	Donar: hydrogen peroxide oxidoreductase	1.10.3.2	Oxidizes phenolic subunits of lignin

Applications in Biotechnology

Production of Fuel Ethanol

Lignocellulosic biomass can serve as low-cost feedstocks for production of fuel ethanol. It generates a mixture of sugars upon pretreatment itself or in combination with enzymatic hydrolysis. The sugar mixture may contain any combination of xylose, arabinose, glucose, galactose, mannose, fucose and rhamnose depending on the source. Although traditional *S. cerevisiae* and *Zymomonas mobilis* ferment glucose to ethanol rapidly and efficiently, they cannot ferment other sugars such as xylose and arabinose to ethanol. Some yeasts (*Pachysolen tannophilus*, *Pichia stipitis*, *Candida shehatae)* have the capability to ferment xylose to ethanol (*143, 144*). These yeasts have low ethanol tolerance and slow rates of fermentation and cannot be used in industrial application (*145, 146*). Xylose can be converted to xylulose using the enzyme xylose isomerase and traditional yeasts can ferment xylulose to ethanol *(147, 148*). However, the process is not cost-effective. Only a few yeast strains can hardly ferment arabinose to ethanol (*149, 150*). Thus, no naturally occurring yeast and bacterium can ferment mixed sugars to ethanol.

Some bacteria such as *Escherichia coli*, *Klebsiella* , *Erwinia*, *Lactobacillus*, *Bacillus* and *Clostridia* can utilize mixed sugars but produce no or limited quantity of ethanol. These bacteria generally produce mixed acids (acetate, lactate, propionate, succinate) and solvents (acetone, butanol, 2,3-butanediol). Several microorganisms have been genetically engineered to produce ethanol from mixed sugar substrates by using two different approaches: (a) divert carbon flow from native fermentation products to ethanol in efficient mixed sugar utilizers such as *Escherichia*, *Erwinia* and *Klebsiella* and (b) introduce the pentose utilizing capability in the efficient ethanol producers such as *Saccharomyces* and *Zymomobilis* (*151-154*). Recombinant *E. coli* K011, *E. coli* SL40, *E. coli* FBR3, *Zymomonas* CP4 (pZB5) and *Saccharomyces* 1400 (pLNH32) strains fermented corn fiber hydrolyzates to ethanol (21-34 g/L) with yields of 0.41-0.50 g of ethanol per gram of sugar consumed (*155-156)*. Increasing gene expression through the replacement of promoters and the use of a higher gene dosage (plasmids) substantially eliminated the apparent requirement for large amounts of complex nutrients of ethanologenic recombinant *E. coli* strain (*157*). Ethanol tolerant mutants of recombinant *E. coli* have been developed that can produce up to 6% ethanol (*158*). The increased ethanol tolerance in the *E. coli* mutant LY01 appears to result from increased glycine metabolism, increased production of the osmolyte betaine from choline, loss of FNR function, increased production of *mar* drug resistance proteins, increased metabolism of serine and pyruvate and decreased production of organic acids (*159*). The recombinant *Z. mobilis* in which four genes from *E. coli*, *xylA* (xylose isomerase), *xylB* (xylulokinase), *tal* (transaldolase) and *tktA* (transketolase) were inserted, grew on xylose as the sole carbon source and produced ethanol at 86% of the theoretical yield (Figure 1) (*153*). It was demonstrated that phosphorylation is a vital step for metabolism of xylose through the pentose phosphate pathway (*160*). The gene *XKS1* (encoding xylulokinase) from *S. cerevisiae* and the heterologous genes from *XYL1* and *XYL2* (from *P. stipitis*) were inserted into a hybrid host, obtained by classical breeding of *S. uvarum* and S. *diastaticus*, which resulted in *Saccharomyces* strain pLNH32, capable of growing on xylose alone. Chromosomal integration of a single copy of the *XYL1-XYL2-XYLS1* cassettee in *S. Cerevisiae* resulted in strain TMB3001 (*161*). This strain attained specific uptake rates (g/g.h) of 0.47 and 0.21 for glucose and xylose, respectively, in continuous culture using a minimal medium. Martin et al. (*162*) studied ethanol production from steam exploded (205 and 215°C, 10 min) enzymatic (cellulase, β-glucosidase) hydrolyzates of sugar cane bagasse using the recombinant *S. cerevisiae* strain TMB 3001. The hydrolyzates were detoxified by treatment with laccase and also by overliming. The ethanol yield was 0.32-0.35 g/g of total sugar from the detoxified hydrolyzates. Partial xylose utilization with low xylitol formation was observed.

Sedlak and Ho (*163*) expressed genes [*arab* (L-ribulokinase), *araA* (L-arabinose isomerase) and *araD* (L-ribulose-5-phosphate)] from the *araBAD* operon encoding the arabinose metabolizing genes from *E. coli* in *S. cerevisiae* but the transformed strain was not able to produce any detectable amount of ethanol from arabinose. Zhang et al. (*164*) constructed one strain of *Z. mobilis* (PZB301) with seven plasmid borne genes encoding xylose- and arabinose metabolizing genes and pentose phosphate pathway (PPP) genes. This recombinant strain was capable of fermenting both xylose and arabinose in a mixture of sugars with 82-84% theoretical yield in 80-100 h at 30°C. Richard et al. (*165*) reported that overexpression of all five enzymes (aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, xylitol dehydrogenase and xylulokinase) of the arabinose catabolic pathway in *S. cerevisiae* led to growth of *S. cerevisiae* on arabinose (Figure 2). The recombinant *S. cerevisiae* produced ethanol from arabinose at a very slow rate.

Microorganisms metabolically engineered with improved inhibitor tolerance could reduce the need for detoxification process. Larsson et al. (*166*) developed a *S. cerevisiae* strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolyzates by heterologous expression of laccase.

Three technology options (concentrated acid process, CHAP; SO_2/dilute acid process, CASH; enzymatic hydrolysis process) were evaluated for ethanol production from pine feedstock using a uniform platform (*167*). Even though each process suffers from certain different disadvantages, none of the three processes can be eliminated as less economical than the other two with an ethanol production price of US $1.89-2.04/gallon. A feasibility study of using softwood forest thinning as a biomass source for ethanol production in California was performed (*168*). A two-stage dilute acid (190°C, 0.7% H_2SO_4, 3 min; 220°C, 1.6% H_2SO_4, 3 min)

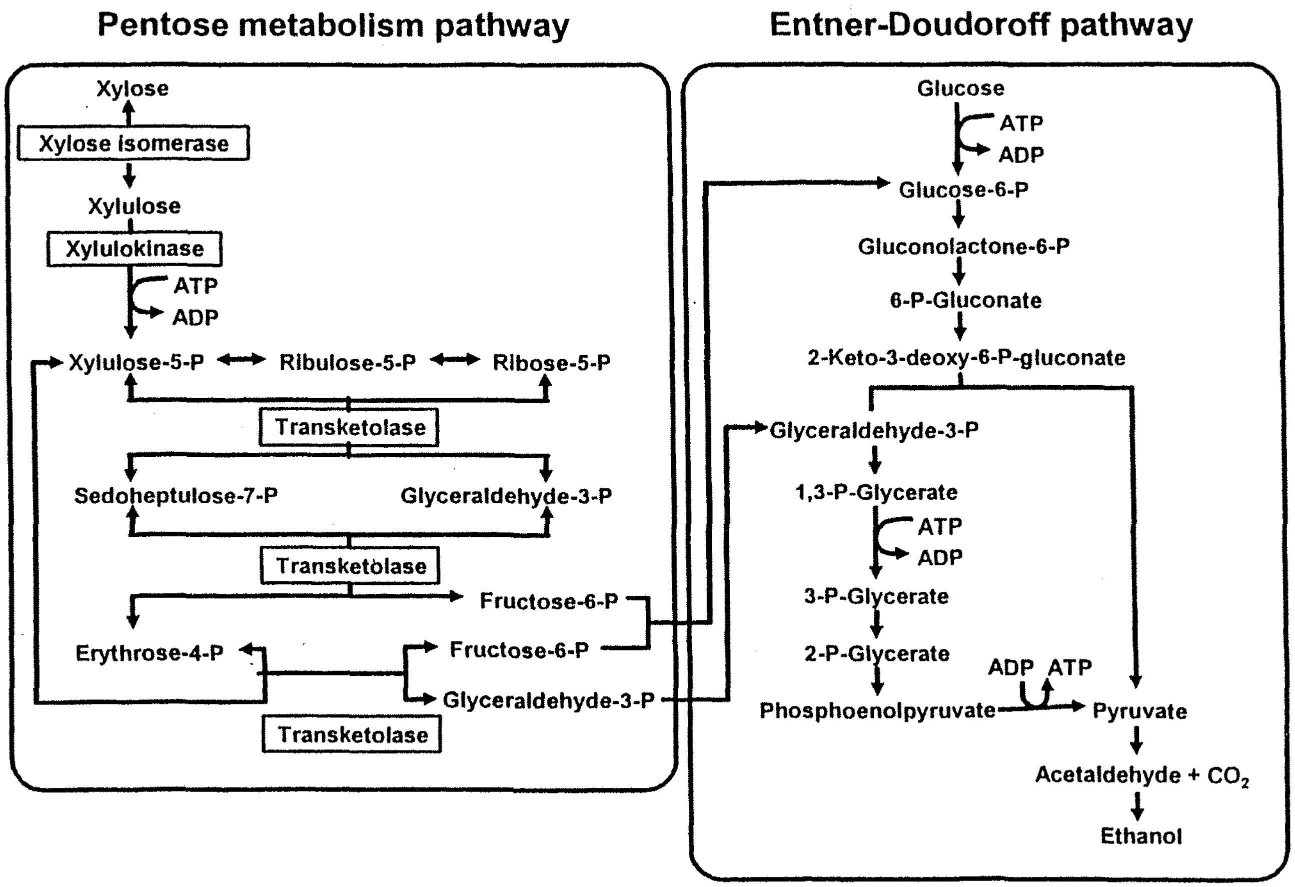
Pentose metabolism pathway
Entner-Doudoroff pathway
Xylose
Xylose isomerase
Xylulose
Xylulokinase
ATP
ADP
Xylulose-5-P
Ribulose-5-P
Ribose-5-P
Transketolase
Sedoheptulose-7-P
Glyceraldehyde-3-P
Transketolase
Fructose-6-P
Erythrose-4-P
Fructose-6-P
Glyceraldehyde-3-P
Transketolase
Glucose
ATP
ADP
Glucose-6-P
Gluconolactone-6-P
6-P-Gluconate
2-Keto-3-deoxy-6-P-gluconate
Glyceraldehyde-3-P
1,3-P-Glycerate
ATP
ADP
3-P-Glycerate
2-P-Glycerate
ADP ATP
Phosphoenolpyruvate
Pyruvate
Acetaldehyde + CO_2
Ethanol

Figure 1. Proposed pentose metabolism and Entner-Doudoroff pathways in engineered Zymomonas mobilis. (Reproduced with permission from reference 153. Copyright 1995.)

hydrolysis process is used for the production of ethanol from softwoods using a recombinant xylose-fermenting yeast, and the residual lignin is used to generate steam and electricity. It is concluded that such a biomass to ethanol plant seems to be an appealing proposition for California, if ethanol replaces MTBE, which is slated for a phase out. With increased research efforts for developing a stable, ethanol tolerant and robust recombinant ethanologenic organism capable of tolerating common fermentation inhibitors generated during pretreatment, competitive and economical production of fuel ethanol from lignocellulose holds strong promise.

Production of Xylitol

Xylitol, a sugar alcohol, has potential use as a natural food sweetener, a dental caries reducer and a sugar substitute for diabetics. It is produced by chemical reduction in alkaline conditions of the xylose derived mainly from wood hydrolyzate (*169*). The recovery of xylitol from the xylan fraction is about 50-60% or 8-15% of the raw material employed. Drawbacks of the chemical process are the requirements of high pressure (up to 50 atm) and temperature (80-140°C), use of an expensive catalyst (Raney-Nickel) and use of extensive separation and purification steps to remove the by-products that are mainly derived from the hemicellulose hydrolyzate (*170*). The bulk of xylitol produced is consumed in various food products such as chewing gum, candy, soft drinks and ice cream. It gives a pleasant cool and fresh sensation due to its high negative heat of solution.

Many yeasts and mycelial fungi possess NADPH dependent xylose reductase (EC 1.1.1.21), which catalyzes the reduction of xylose to xylitol as a first step in xylose metabolism (*171*). Xylitol can be subsequently oxidized to xylulose by the action of xylitol dehydrogenase, which preferentially uses NAD as an acceptor (*172*). In xylose fermenting yeasts, the initial reactions of xylose metabolism appear to be rate-limiting (*173*). This results in accumulation of xylitol in the culture medium, the degree varying with the culture conditions and the yeast strain used (*174*). A surplus of NADH during transient oxygen limitation inhibiting the activity of xylitol dehydrogenase results in xylitol accumulation (*175*). The pathway for xylose utilization in microorganisms is shown in Figure 3. Some of the natural xylose-fermenting yeasts that are known to produce xylitol are: *C. boidini*, *C. entomaea*, *C. guillermondii*, *C. peltata* , *C. tropicalis*, *C. parapsilosis* and *Debaryomyces hansenii* (*176-179*). Oxygen plays an important role in xylose uptake by yeasts. Phosphate limitation stress induces xylitol overproduction by *D. hansenii* (*180*). The highest xylitol concentration attained in microbial processes using xylose as substrate have been in the range of 200 to 220 g/L (*181-184*). Nakano et al. (*185*) reported very high xylitol (356 g/L) production by *C. magnoliae* in fed-batch culture under a microaerobic condition maintained by

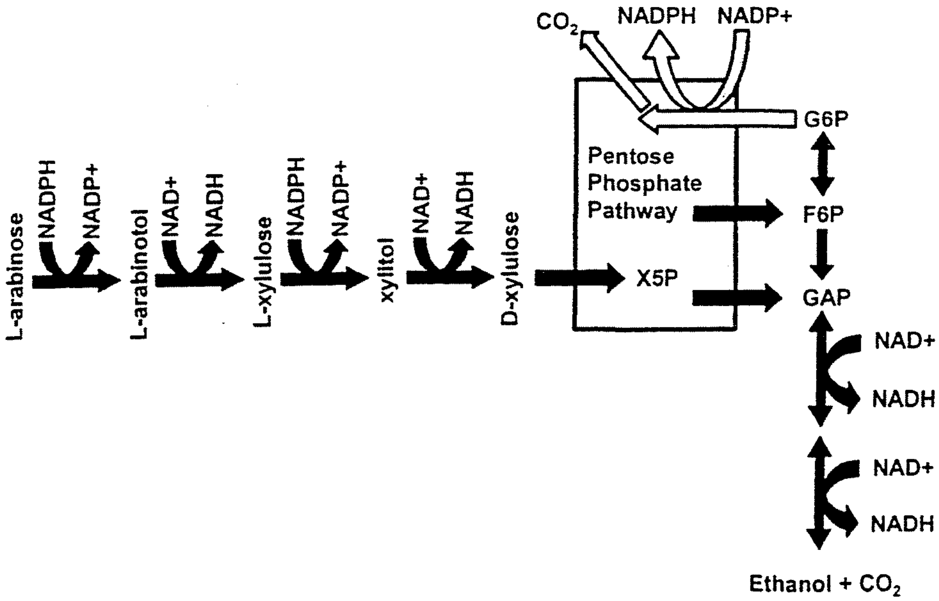
CO2
NADPH
NADP+
G6P
Pentose Phosphate Pathway
F6P
X5P
GAP
L-arabinose
NADPH
NADP+
L-arabinotol
NAD+
NADH
L-xylulose
NADPH
NADP+
xylitol
NAD+
NADH
D-xylulose
NAD+
NADH
NAD+
NADH
Ethanol + CO2

Figure 2. Redox cofactor requirement in L-arabinose catabolism. L-Arabinose conversion to equimolar amounts of CO_2 and ethanol is redox neutral, i.e. anaerobic fermentation to ethanol should be possible. However, the conversion of L-arabinose to D-xylulose requires NADPH and NAD^+ and produces NADH and $NADP^+$. NADPH is mainly regenerated in the oxidative part of the pentose phosphate pathway, where the reduction of $NADP^+$ is coupled to CO2 production. The abbreviations are: G6p, glucose-6-phosphate; F6P, fructose 6-phosphate; X5P, D-Xylulose 5-phosphate; GAP, D-glyceraldehyde 3-phosphate. (Reproduced from Ref. *165* with permission from Elsevier Science)

simple fuzzy control with a yield of 0.75, which corresponded to 82% of the theoretical yield..

The fermentation of sugarcane bagasse hemicellulose hydrolyzate to xylitol by a hydrolyzate-acclimatized yeast strain *Candida* sp. B-22 was studied (*186*). A final xylitol concentration of 94.74 g/L was obtained from 105.35 g/L xylose in hemicellulose hydrolyzate after 96 h of incubation. *C. guilliermondii* FTI 20037 was able to ferment a sugar cane bagasse hydrolyzate producing 18.4 g/L xylitol from 29.5 g/L of xylose, at a production rate of 0.38 g/L.h (*187*). This lower value, compared to that (0.66 g/L.h) of the synthetic medium, may be attributed to the various toxic substances that interfere with microbial metabolism (e.g., acetic acid). Dominguez et al. (*188*) studied different treatments (neutralization, activated charcoal and neutralization, cation-exchange resins and neutralization) of sugar cane bagasse hemicellulose hydrolyzate to overcome the inhibitory effect on xylitol production by *Candida* sp. 11-2. The highest xylitol productivity (0.205 g/L.h), corresponding to 10.54 g/L, was obtained from hydrolyzates treated with activated charcoal (initial xylose, 42.96 g/L). To obtain higher xylitol productivity, treated hydrolyzates were concentrated by vacuum evaporation in rotavator to provide higher initial xylose concentration. The rate of xylitol production increased with increasing initial xylose concentration from 30 to 50 g/L, reaching a maximum of 28.9 g/L after 48 h fermentation. The decrease in xylitol production was dramatic with further increases in the initial xylose concentration. Parajo et al. (*189*) reported a xylitol production of 39-41 g/L from concentrated *Eucalyptus globulus* wood acid hydrolyzate containing 58-78 g xylose/L by *D. hansenii* NRRL Y-7426 using an initial cell concentration of 50-80 g/L. Recently, Rivas et al (*190*) achieved a xylitol concentration of 71 g/L and volumetric productivity of 1.5 g/L.h when *D. hansenii* NRRL Y-7426 (12 g dry mass/L) was grown semiaerobically using detoxified corn cob hydrolyzate produced by autohydrolysis-posthydrolysis at starting xylose concentration of 100 g/L.

Hydrolyzed hemicellulosic fractions of sugar cane bagasse and rice straw were tested for xylitol production in batch fermentation by *C. guilliermondii* under semiaerobic condition and compared these with synthetic medium containing xylose (*191*, *192*). Simultaneous utilization of hemicellulosic sugars (glucose and xylose) was observed, and the highest substrate uptake rate was attained in sugar cane bagasse medium. Increased xylitol concentration (40 g/L) was achieved in synthetic and rice straw media, although the highest xylitol production rate was obtained in sugar cane bagasse hydrolyzate. Both hydrolyzates can be converted into xylitol with satisfactory yields and productivities. Xylitol production by *C. guilliermondii* was evaluated using rice straw hemicellulose hydrolyzate under different conditions of initial pH, nitrogen sources and inoculum level (*193,194*). The xylitol yields were 0.68 g/g for the medium containing ammonium sulfate at pH 5.3 and 0.66 g/g with urea at pH 4.5. Under appropriate inoculum conditions, rice straw hemicellulose hydrolyzate was converted into xylitol by the yeast with 77% yield. Mayerhoff et al. (*195*) evaluated 30 different yeast strains belonging to 4 different genera (*Candida*, *Debaryomyces*, *Hansenula* and *Pichia*) for xylitol production from rice straw hemicellulose hydrolyzate. The best performer was *C. mogii* NRRL Y-17032, which yielded 0.65 g xylitol/g at 0.40 g/L.h over 75 h. Preziosi-Belloy et al. (*196*) investigated the production of xylitol from aspenwood hemicellulose hydrolyzate by *C. guilliermondii*. The hydrolyzate was supplemented by yeast extract, and the maximum xylitol yield (0.8 g/g) and productivity (0.6 g/L.h) were reached by controlling oxygen input. A two-stage sequential fermentation scheme for production of xylitol and arabitol from a mixture of sugars by *C. guilliermondii* was developed (*197*). Following glucose consumption, cells

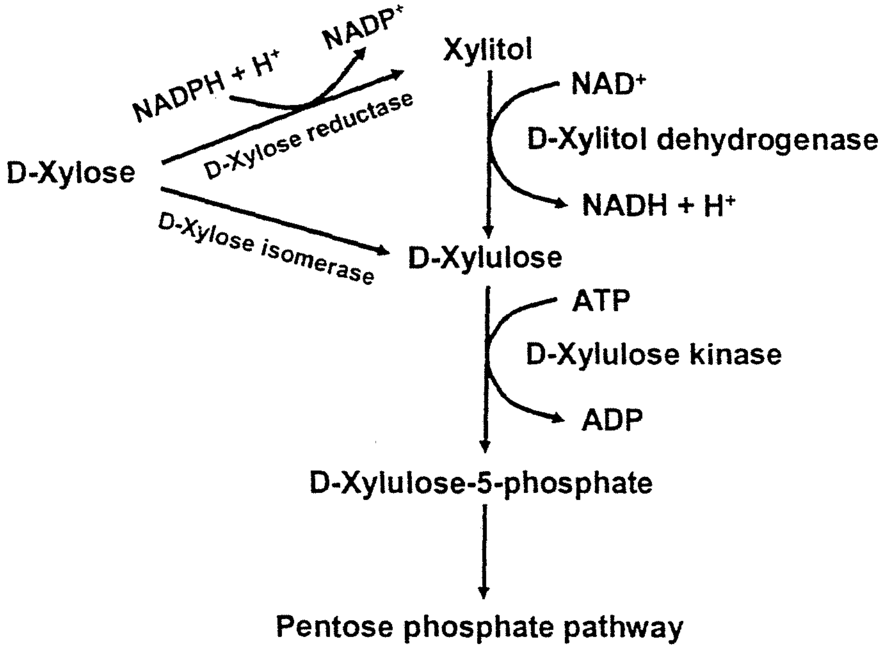
NADPH + H+
NADP+
Xylitol
D-Xylose reductase
D-Xylose
D-Xylose isomerase
D-Xylulose
NAD+
D-Xylitol dehydrogenase
NADH + H+
ATP
D-Xylulose kinase
ADP
D-Xylulose-5-phosphate
Pentose phosphate pathway

Figure 3. Pathway for xylose utilization in microorganisms. (Reproduced from reference 176. Copyright 1997 American Chemical Society.)

were removed from mixed sugar cultures and replaced with cells from cultures grown on xylose alone. In the second fermentation stage, xylose and arabinose were successfully fermented to xylitol and arabitol. Dilute acid hydrolyzate of corn fiber after treatment with a mixed-bed deionization resin was suitable for the two-stage fermentation process. Xylitol production was studied from barley bran hydrolyzates by continuous fermentation with *D. hansenii* (*198*). The optimum xylitol productivity (2.53 g/L.h) was reached at a dilution of rate of 0.284/h with cell recycle after membrane separation. Xylitol was produced in a two-substrate (xylose, glucose) batch fermentation by *C. tropicalis* with cell recycling (*199*). The optimized cell recycle fermentation resulted in xylitol yield of 0.823 g/g xylose with a productivity of 4.94 g/L.h and a final xylitol concentration of 189 g/L. Xylitol productivity up to 1.14 g xylitol/L.h with 86% conversion efficiency was achieved with a strain of *C. guilliermondii* in continuous culture using a membrane bioreactor at a dilution rate of 0.03/h (*200*).

Carvalho et al. (*201*) achieved maximum xylitol concentration of 20.6 g/L with a volumetric productivity of 0.43 g/L.h and yield of 0.47 g/g after 48 h fermentation during batch xylitol production from concentrated sugarcane bagasse hydrolyzate by *C. guilliermondii* cells, immobilized in calcium-alginate beads. Aeration rate strongly influences xylitol production from sugarcane bagasse hydrolyzate by immobilized cells of *C. guilliermondii* in a fluidized bed reactor (*202*).

E. coli JM 109 was used as a host for xylitol production by expressing xylose reductase gene (*xyrA*) of *C. tropicalis* IFO 0618 (*203*). When xylose (50 g/L) and glucose (5 g/L) were added to IPTG-induced cells, 13.3 g/L of xylitol was produced during 20 h of cultivation. A number of recombinant *S. cerevisiae* strains have been created by expressing the xylose reductase gene (*XYL1*) from *P. stipitis* and *C. shehate,* and production of xylitol from xylose by these recombinant strains in batch and fed batch fermentations have been investigated (*204-206*). Lee et al. (*207*) studied the fermentation characteristics of recombinant *S. ceresevíae* containing a xylose reductase gene from *P. stipitis*. Xylitol was produced with a maximum yield of 0.95 g/g xylose consumed in the presence of glucose used as co-substrate for co-factor regeneration. However, addition of glucose caused inhibition of xylose transport and accumulation of ethanol. By adopting a glucose-limited fed batch fermentation where a high ratio of xylose to glucose was maintained, a xylitol concentration of 105.2 g/L was achieved with a 1.69 g/l.h productivity with this recombinant yeast. Kim et al. (*208*) reported that *S. cerevisiae* containing multiple XYL1 genes of *P. stipitis* on the chromosome is much more efficient for xylitol production in the long term non-selective culture than S. *cerevisiae* harboring the episomal plasmid containing the XYL1 gene. Such an improvement in the integrated recombinant strain was supported by the fact that the mitotic stability of the XR gene along with its high expression level worked in a cooperative manner (*209*).

Continuous enzymatic production of xylitol with simultaneous coenzyme regeneration in a charged membrane reactor was studied (*210*). An NADH dependent xylose reductase from *C. tenuis* catalyzed the reduction of xylose. This was coupled to enzymatic oxidation of glucose by glucose dehydrogenase from *Bacillus cereus* to make achievable an up to 10,000-fold regeneration of NADH per cycle of discontinuous conversion. Under suitable conditions, 300 g/L of substrate could be converted in yields above 96% in one single batch reaction.

The recovery of xylitol from fermented sugarcane bagasse hydrolyzate was studied (*211*). The best clarifying treatment was found by adding 20 g activated carbon to 100 ml fermented broth at 80°C for 1 h at pH 6.0. The clarified medium was treated with ion exchange resins after which xylitol crystallization was attempted. The ion exchange resins were not efficient but the crystallization technique showed good performance, although the crystals were involved in a viscous and colored solution. Recently, Faveri et al. (*212*) reported xylitol recovery by crystallization from synthetic solutions and fermented hemicellulose hydrolyzates. The method involves evaporation of dilute solution up to super saturation, cooling of super saturation solution, separation of crystals by centrifugation and final filtration. Using two sets of tests on xylitol-xylose synthetic solutions and one set on fermented hardwood hemicellulose hydrolyzate, the best results in terms of either crystallization (0.56) yield or purity degree (1.00) were obtained with quite concentrated solutions of 730 g/L at relatively high temperature

(-5°C). They concluded that xylitol separation by crystallization from fermented hemicellulose hydrolyzate is feasible.

Thus, xylose rich hemicellulosic materials can serve as abundant and cheap feedstocks for production of xylitol by fermentation. It is possible to introduce the pathway for conversion of arabinose to xylitol in the xylitol producing yeast. In that case, xylitol can be produced from both xylose and arabinose.

Production of 2,3-butanediol

2,3-Butanediol, otherwise known as 2,3-butylene glycol (2,3-BD), is a valuable chemical feedstock because of its application as a solvent, liquid fuel and as a precursor of many synthetic polymers and resins. With a heating value of 27,200 J/g, 2,3-BD compares favorably with ethanol (29,100 J/g) and methanol (22,100 J/g) for use as a liquid fuel and fuel additive (*213*). Dehydration of 2,3-BD yieldsthe industrial solvent methyl ethyl ketone which is much more suited as a fuel because of its much lower boiling point. Further dehydration yields 1,3-butanediene, which is the starting material for synthetic rubber and is also an important monomer in the polymer industry (*214*). During World War II, it was needed for conversion to 1,3-butanediene. Methyl ethyl ketone can be hydrogenated to yield high octane isomers suitable for high quality aviation fuels. Diacetyl, formed by catalytic dehydrogenation of the diol, is a highly valued food additive (*215*). A wide variety of chemicals can also be easily prepared from 2,3-BD (*216, 217*). There is an interest in industrial scale production of 2,3-BD from various agricultural residues as well as logging, pulp and paper, and food industry wastes (*215*).

2,3-BD can occur in two enantiomeric forms: D- (-) and L-(+) as well as an optically inactive meso-form. *B. polymyxa* produces D-(-) 2,3-BD whereas *Klebsiella pneumoniae* (*Aerobacter aerogenes*) produce meso-form and also some of the L-(+) form. *B. subtilis*, *Seratia marcescens* and *A. hydrophia* produce mixtures of different forms (*218*). A newly isolated *Enterobacter cloacae* NRRL B-23289 produces meso-2,3-butanediol (0.35-0.43 g/g sugar) from a variety of sugar substrates including corn fiber hydrolyzates (*219*). The typical 2,3-BD yield was 0.30-0.45 g/g sugar (*214, 220*). Ui et al. (*221*) cloned a gene fragment including genes coding for three enzymes (α-acetolactate synthase, α-acetolactate decarboxylase and meso2,3-BD dehydrogenase (D-acetoin forming) involved in the formation of meso-BD of *K. pneumoniae* IAM 1063 in *E. coli* JM 109 after its insertion into pUC118. The resulting *E. coli* JM109/pBDO118 produced 17.7 g of meso-BD from 100 g of glucose per L.

Butanediol is produced during oxygen limited growth by a fermentative pathway known as mixed acid-butanediol pathway (Figure 4) (*222*). The 2,3-BD pathway and the relative proportions of acetoin and butanediol serve to maintain the intracellular NAD/NADH balance in changing culture conditions. The theoretical maximum yield of 2,3-BD from monosaccharides is 0.5 g/g (*223*). The efficient biological conversion of all the available sugars in agricultural biomass residues to fuels and chemicals is crucial to the efficiency of any process intended to compete economically with petrochemical products (*217*).

The high boiling point of 2,3-BD, its high affinity for water, and the dissolved and solid substances of the fermentation broth make it difficult for 2,3-BD to be purified and recovered from fermentation slurry (*224*). Various methods such as solvent extraction, liquid-liquid extraction and salting out have been used to recover butanediol. Another feasible method to recover butanediol is by countercurrent stream stripping (*225*).

Production of Vanillin

Ferulic acid is the major cinnamic acid found in a variety of plant cell walls. Corn fiber contains about 3% ferulic acid. Wheat bran is another source of ferulic acid (0.5-1%). Faulds et al. (*226*) developed a laboratory scale procedure to produce free ferulic acid (5.7 g) from wheat bran (1 kg) by using a *Trichoderma* xylanase preparation and *A. niger* ferulic acid esterase. Using filamentous fungi, a two-stage process for vanillin formation was developed in which a strain of *A. niger* was first used to convert ferulic acid to vanillic acid, which was then reduced to vanillin by a laccase-deficient strain of *Pycnoporus cinnabarinus* (*227*).

Concluding Remarks

Lignocellulose biodegradation and its conversion to a wide variety of commodity chemicals holds enormous potential. At present, the conversion of lignocellulosic biomass to fermentable sugars is not cost-effective. Some of the emerging pretreatment methods such as alkaline peroxide and AFEX generate solubilized and partially degraded hemicellulosic biomass that need to be treated further with enzymes or other means to produce fermentable sugars from them. With the development of a suitable pretreatment method minimizing the formation of inhibitory compounds for fermentative organisms and use of proper mixture of cellulases and hemicellulases (enzyme cocktail) tailored for each biomass conversion, this vast renewable resource can be utilized for production of fuels and chemicals by fermentation or enzymatic means. Research emphasis should be to develop efficient and cost-effective pretreatment method, enzymes for use in cellulose and hemicellulose conversion in an industrial scale, robust efficient microorganism to ferment lignocellulosic hydrolyzates in a cost-competitive way and method for cost-effective recovery of fermentation products. Finally, integration of various process steps such as biomass pretreatment, enzymatic

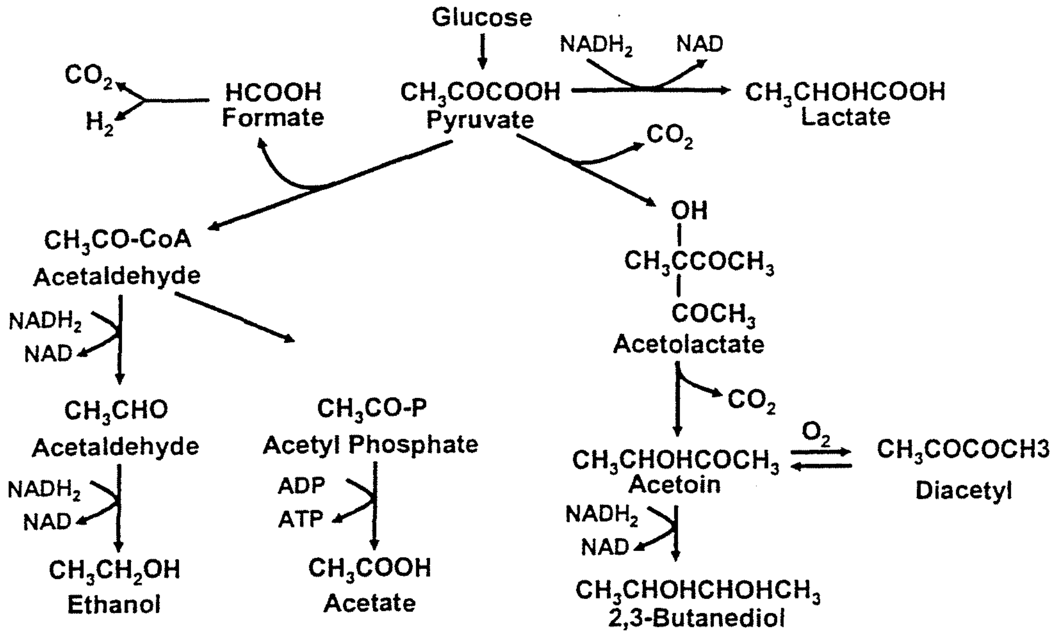
Glucose
NADH$_2$
NAD
CO_2
H_2
HCOOH
Formate
$CH_3COCOOH$
Pyruvate
$CH_3CHOHCOOH$
Lactate
CO_2
$CH_3CO\text{-}CoA$
Acetaldehyde
OH
CH_3CCOCH_3
$COCH_3$
Acetolactate
NADH$_2$
NAD
CO_2
CH_3CHO
Acetaldehyde
$CH_3CO\text{-}P$
Acetyl Phosphate
O_2
$CH_3CHOHCOCH_3$
Acetoin
$CH_3COCOCH3$
Diacetyl
NADH$_2$
NAD
ADP
ATP
NADH$_2$
NAD
CH_3CH_2OH
Ethanol
CH_3COOH
Acetate
$CH_3CHOHCHOHCH_3$
2,3-Butanediol

Figure 4. Metabolic pathway of 2,3-butanediol production from glucose.

saccharification, detoxification, fermentation of the hydrolyzates and recovery of products will greatly aid in reducing the overall cost of using lignocellulose for practical purposes.

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